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Molecules of Interest

Molecular basis for cytokinin biosynthesis

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ABSTRACT

Cytokinins (CKs) are a group of phytohormones that play a crucial role in the regulation of plant growth and development. Identification of the enzymes and the corresponding genes that are involved in CK metabolism allowed us to understand how plants synthesize CKs and adjust CK activity to optimal levels. A major accomplishment toward these goals was the identification of genes for the first enzyme in the CK biosynthetic pathway, adenosine phosphate-isopentenyltransferase (IPT). In *Arabidopsis thaliana* and *Agrobacterium tumefaciens*, detailed analyses of IPTs were conducted through not only enzymatic characterization but also molecular structural approaches. These studies revealed the molecular basis for the *Agrobacterium*-origin of IPT used for the efficient biosynthesis of *trans*-zeatin that promotes tumorigenesis in host plants. Another landmark in CK research was the identification of CYP735A as an enzyme that converts iP-nucleotide to tZ-nucleotide. Furthermore, the identification of a CK-activating enzyme, LOG, which catalyzes a novel activation pathway, is a remarkable recent achievement in CK research. Collectively, these advances have revealed the complexity of the entire metabolic scheme for CK biosynthesis.

1. Introduction

Cytokinins (CKs) are a group of phytohormones that are involved in a wide variety of plant growth and development processes, such as delay of senescence (Gan and Amasino, 1995; Kim et al., 2006), root proliferation (Werner et al., 2003, 2001), apical dominance (Sachs and Thimann, 1967; Shimizu-Sato et al., 2009; Tanaka et al., 2006), nutritional signaling (Samuelson and Larsson, 1993; Takei et al., 2001b) and shoot meristem function (Higuchi et al., 2004; Kurakawa et al., 2007; Nishimura et al., 2004).

Naturally occurring CKs are adenine derivatives that carry either an isoprene-derived side chain or an aromatic side chain at the N^6-terminus, called isoprenoid CKs or aromatic CKs, respectively (Mok and Mok, 2001; Sakakibara, 2006). Because isoprenoid CKs are commonly found in higher plants and are much more abundant than aromatic ones, the metabolic pathway for the isoprenoid CKs has been studied with great intensity. On the other hand, the biosynthetic pathway for the aromatic CKs has not been elucidated, even though these forms of CKs are found in some higher plants (Strnad, 1997), mosses (von Schwartzenberg et al., 2007) and unicellular algae (Ordog et al., 2004).

Although many studies attempting to purify and characterize plant CK biosynthetic enzymes have been conducted, only a few biochemical properties have been reported (Mok and Mok, 2001; Sakakibara, 2004), probably due to enzyme instability or the small amount of enzyme present in plant cells. However, the accumulation of genomic information in various organisms and advances in technologies for plant hormone analysis provided several breakthroughs for elucidating CK metabolism, as well as its signaling system. Among the most important advances are the identification of genes involved in CK biosynthesis in model plants, such as *Arabidopsis* and rice (Kakimoto, 2001; Kurakawa et al., 2007; Sakamoto et al., 2006; Takei et al., 2001a, 2004b), determination of crystal structures of enzymes catalyzing CK biosynthesis (Sugawara et al., 2008) and degradation (Malito et al., 2004), and discovery of a novel pathway for the production of active CK species (Kurakawa et al., 2007). Furthermore, advances in studies on CK production by phytopathogenic bacteria revealed that an *Agrobacterium*-origin enzyme efficiently produced CK for tumorigenesis by utilizing substrates distinct from those used by the host plant enzyme (Sakakibara et al., 2005). In this review, we focus on the enzymes that function in isoprenoid CK biosynthesis, and outline the biosynthetic pathways and the molecular mechanisms for CK biosynthesis reactions and preferential substrate use.

2. Structural variation in CKs

Natural isoprenoid CKs are classified into one of four basic molecules: N^6-(Δ^2-isopentenyl)adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ) and dihydrozeatin (DZ) (Fig. 1). Each CK molecule is distinguished by characteristics of the side chain, namely the presence or the absence of a hydroxyl group at the end of the prenyl chain and the stereoisomeric position. Although there is no widely

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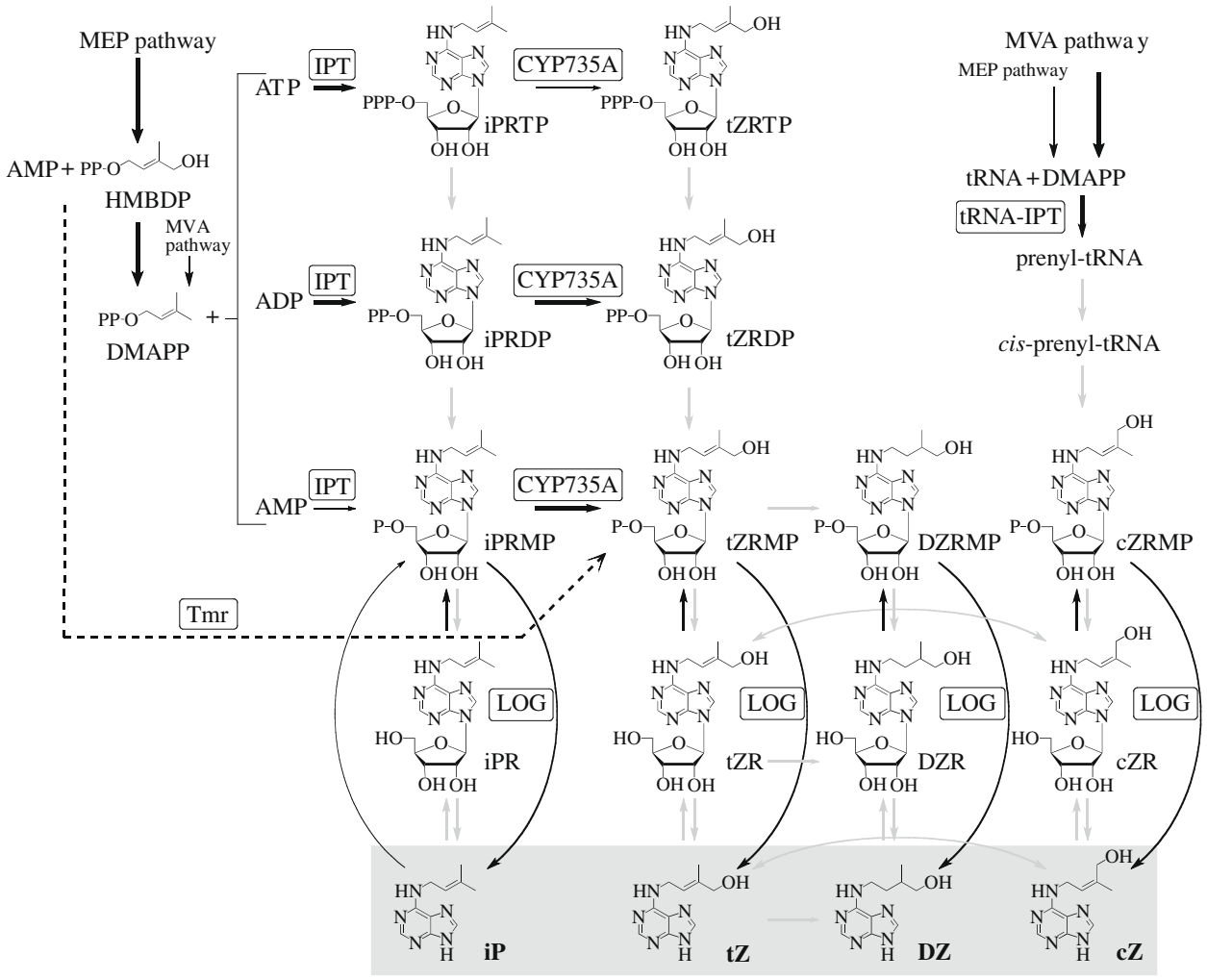


Fig. 1. Current model of isoprenoid CK biosynthetic pathways in higher plants and in infected plant cells by *A. tumefaciens*. The isoprenoid side chain of iP and tZ predominantly comes from the MEP pathway, whereas that of cZ mostly originates from the MVA pathway. Adenosine phosphate-isopentenyltransferases (IPTs) in higher plants preferably utilizes ATP and ADP as isoprenoid acceptors and catalyze *N*-prenylation using DMAPP, producing iPRTP and iPRDP, respectively. The CK-nucleotides are converted into the corresponding tZ-nucleotides by CYP735A, which prefer iPRDT and iPRMP more than iPRTP. Dephosphorylation by phosphatase may occur in those di- or tri-phosphorylated CK-nucleosides. In cells infected by *A. tumefaciens*, Tmr encoded by the bacterium mediates *N*-prenylation of AMP with HMBDP, producing tZRMP (dashed arrow line). The tRNA-IPTs catalyze prenylation of tRNA that leads to the production of cZRMP. The CK-nucleoside 5′-monophosphates are directly catalyzed to active form (free-base form) CKs by LOG. cZ and tZ may be enzymatically interconverted by zeatin *cis–trans* isomerase. The genes of enzymes involved in phosphorylation of iPR and conjugation of phosphoribosyl moieties to iP are adenosine kinase (AK) and adenine phosphoribosyltransferase (APRT), respectively. APRT utilizes not only iP but also other CK nucleobases. The width of the lines indicates the strength of metabolic flow. Gray arrows show the flow in which the related enzymes have not yet been identified. In chemical formulae, phosphate is abbreviated to P.

accepted explanation for the physiological relevance of these structural variants at present, differences have been found in their activity and stability *in vivo*. The relative biological activities of CKs were estimated by measuring their ligand affinity to CK receptors. *In vitro* and *in vivo* studies with Arabidopsis CK receptors (AHK3 and AHK4/WOL/CRE1) showed that iP and tZ have much higher affinities than cZ and DZ (Inoue et al., 2001; Romanov et al., 2006; Spichal et al., 2004; Suzuki et al., 2001; Yamada et al., 2001). Among the CK-nucleobases tZ, iP, and cZ, tZ was the most active and cZ was the least active form in bioassays using cucumber and *Amaranthus caudatus* (Kaminek et al., 1979). On the other hand, a CK receptor from maize (ZmHK1) responded to cZ and tZ with similar sensitivity (Yonekura-Sakakibara et al., 2004), inferring that the relative activities of CKs might vary among plant species. Susceptibility to cytokinin oxidase (CKX), a CK-degrading enzyme, is also different among the CKs (Bilyeu et al., 2001; Galuszka et al., 2007). Generally, iP is more susceptible to CKX than tZ and cZ, and DZ is resistant to CKX.

3. Adenosine phosphate-isopentenyltransferases (IPTs)

The first step of isoprenoid CK biosynthesis is catalyzed by adenosine phosphate-isopentenyltransferase (IPT; EC 2.5.1.27) that catalyzes the *N*-prenylation of adenosine 5′-phosphate (AMP, ADP or ATP) at the N^6-terminus with dimethylallyl diphosphate (DMAPP) or 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (HMBDP) (Fig. 1). IPTs have been identified in higher plants (Kakimoto, 2001; Sakamoto et al., 2006; Sakano et al., 2004; Takei et al., 2001a; Zubko et al., 2002) and some phytopathogenic microorganisms such as *Agrobacterium tumefaciens* (Akiyoshi et al., 1984; Barry et al., 1984) and *Rhodococcus fascians* (Crespi et al., 1992).

In *Arabidopsis thaliana* and rice, seven IPT genes (*AtIPT1*, *AtIPT3*–*AtIPT8*) (Kakimoto, 2001; Takei et al., 2001a) and eight IPT genes (*OsIPT1*–*OsIPT8*) (Sakamoto et al., 2006) were identified as being involved in the *N*-prenylation step of CK biosynthesis, respectively, although some sequences (*AtIPT6* and *OsIPT6*) seem to be pseudo-genes in some cultivars. AtIPTs and OsIPTs prefer ATP or ADP

to AMP as the prenyl acceptor, and utilize DMAPP as the prenyl-donor (Kakimoto, 2001; Sakamoto et al., 2006) (Fig. 1). The reaction produces iP-nucleotides, specifically iP riboside 5′-triphosphate (iPRTP) and iP riboside 5′-diphosphate (iPRDP) using ATP and ADP, respectively. The K_m values of AtIPT1 for ATP, ADP and AMP were 11.4, 14.6 and 185 μM, respectively. The K_m for DMAPP was 8.3 μM, but the K_m for HMBDP could not determined due to very low affinity (Sakakibara, 2004; Sakakibara et al., 2005; Takei et al., 2001a).

The prenyl-donor for the IPT reaction, DMAPP, can be produced by both the methylerythritol phosphate (MEP) pathway in plastids and the mevalonate (MVA) pathway in the cytosol. The prenyl group of tZ and iP is mainly produced by the MEP pathway as shown by isotope-labeling experiments in *Arabidopsis* seedlings (Kasahara et al., 2004) (Fig. 1), suggesting that plastids play an important role in the initial step of CK biosynthesis in *Arabidopsis*. In concurrence with this result, four AtIPTs (AtIPT1, AtIPT3, AtIPT5, AtIPT8) were localized in plastids (Kasahara et al., 2004), and *AtIPT3* and *AtIPT5* show relative higher levels of expression than the others (Miyawaki et al., 2004; Takei et al., 2004a). In contrast, AtIPT4 and AtIPT7 localize to the cytosol and mitochondria, respectively (Kasahara et al., 2004). There are two reports that inhibition of the MVA pathway by an inhibitor, lovastatin, significantly decreased CK accumulation in tobacco BY-2 cells (Laureys et al., 1998, 1999), suggesting that the MVA pathway also provides DMAPP for CK biosynthesis in some situations.

Unlike AtIPTs and OsIPTs, *Agrobacterium* IPTs, Tmr and Tzs, catalyze the *N*-prenylation of AMP using DMAPP or HMBDP as an isoprene donor *in vitro* (Blackwell and Horgan, 1993; Krall et al., 2002; Sakakibara et al., 2005; Sugawara et al., 2008). The bacterial enzymes do not utilize ATP or ADP. The K_m values of Tmr and Tzs for DMAPP and HMBDP are similar (Tmr for DMAPP; 10.1 μM and for HMBDP; 13.6 μM, Tzs for DMAPP; 7.9 μM and for HMBDP; 8.2 μM) (Sakakibara et al., 2005; Sugawara et al., 2008). Thus, the *Agrobacterium* IPTs can utilize both substrates with (HMBDP) or without (DMAPP) an hydroxylated-prenyl side chain, producing tZ riboside 5′-phosphate (tZRMP) or iP riboside 5′-phosphate (iPRMP), respectively. Isotope-tracer experiments verified that HMBDP, not DMAPP, was the major substrate of Tmr *in planta* after infection by *A. tumefaciens* (Sakakibara et al., 2005).

HMBDP is an intermediate product of the MEP pathway in plastids (Fig. 1). In concert with the use of HMBDP as the principal substrate, Tmr localizes to plastids after infection, although the mechanism is unknown (Sakakibara et al., 2005). These results strongly suggest that tZRMP is directly produced in *A. tumefaciens*-infected plant cells by Tmr function (Fig. 1). In fact, crown galls and *Tmr*-overexpressing transgenic plants almost exclusively contain tZ-type CKs (Faiss et al., 1997; Morris, 1986; Sakakibara et al., 2005), implying an unknown mechanism for preferential usage of HMBDP by Tmr *in planta*. Thus, Tmr successfully competes against the host plant IPTs for substrates in the plastids of *A. tumefaciens*-infected plant cells due to its greater substrate specificity.

4. Structural basis for IPT activity in cytokinin biosynthesis

Determining the crystal structures of Tzs complexed with substrates or their analogues provided us with insights into the reaction mechanism for the initial step of CK biosynthesis (Sugawara et al., 2008). The carbon–nitrogen-based prenylation proceeds *via* an SN2-reaction mechanism. Tzs has a solvent-accessible channel that is the reaction center (Fig. 2A). The prenyl-donor substrate, DMAPP or HMBDP, is fixed with amino acid residues of a p-loop (Gly8-to-Thr15 in Tzs). In the reaction, the carboxylate group of an Asp residue (Asp33 in Tzs) serves as a general base to deprotonate the N^6-amino group of AMP (Fig. 2B). The resulting nucleophile attacks the carbon (C^1) of DMAPP. Collapse of the penta-covalent transition state yields the products iP riboside 5′-monophosphate and diphosphate. Thr and Arg residues (Thr10 and Arg138 in Tzs, respectively) stabilize the penta-covalent transition state. IPT requires a divalent metal ion, such as Mg^{2+}. The metal ion plays a role in neutralizing the negative charge of the diphosphate and in fixing the prenyl-donor substrate to undergo the catalytic reaction.

Crystal structure determination also enabled us to identify critical amino acid residues ruling prenyl-donor substrate specificity between plant and bacterial IPTs (Sugawara et al., 2008). Introductions of amino acid substitutions into a hydrophilic region on the surface of the reaction cavity (Glu210 to His214 and Asp173 in Tzs) surrounding the *trans*-side end of the dimethylallyl group (Fig. 3A) drastically changed the kinetic parameters (Sugawara et al., 2008). His214 and Asp173 in Tzs are also conserved in Tmr, but not in higher plant IPTs (Fig. 3B). Substitution of His214 with Leu or Asp173 with Gly greatly reduced affinity to HMBDP but not to DMAPP, indicating that the hydrophilic region formed by His214 and Asp173 crucially affects the specificity of the prenyl-donor in Tzs. These results suggest that differences in these amino acids between *Agrobacterium* and *Arabidopsis* allow *Agrobacterium*-origin IPT to use HMBDP for direct synthesis of tZ, a highly active CK.

5. tRNA-isopentenyltransferases (tRNA-IPTs)

Another pathway for biosynthesis of isoprenoid CK is tRNA prenylation catalyzed by tRNA-isopentenyltransferase (tRNA-IPT; EC 2.5.1.8) (Fig. 1). Some tRNA species can undergo prenylation at the adenine adjacent to the 3′-end of the anticodon by tRNA-IPT (Murai, 1994). Prenylated-tRNA has a *cis*-hydroxyl group, thus degradation of prenylated tRNA generates cZ. tRNA-IPT is widely found in diverse organisms, from bacteria to animals and plants.

Arabidopsis and rice have two genes for tRNA-IPT isozymes, *AtIPT2* and *AtIPT9* in *Arabidopsis* (Miyawaki et al., 2006), and *OsIPT9* and *OsIPT10* in rice (Sakamoto et al., 2006). In *Arabidopsis*, a mutant deficient in both tRNA-IPTs resulted in the cZ content to fall below detection limits, although the levels of iP- and tZ-type CKs were unaffected (Miyawaki et al., 2006). This report suggested that prenylated tRNA-degradation is the main pathway to supply cZ in *Arabidopsis*. Contrary to that assertion, the prenyl group of tZ and iP is mainly produced through the MEP pathway by the function of IPTs, a substantial part of cZ comes from the MVA pathway in *Arabidopsis* (Kasahara et al., 2004) (Fig. 1). In fact, a tRNA-IPT in *Arabidopsis*, AtIPT2, localizes in the cytosol (Kasahara et al., 2004).

Recent structural studies on tRNA-IPTs from *Pseudomonas aeruginosa* (Xie et al., 2007) and *Saccharomyces cerevisiae* (Zhou and Huang, 2008) have revealed that IPT and tRNA-IPTs share a conserved reaction mechanism and critical amino acid residues. Because tRNA-IPT occurs in a wide variety of organisms other than those of the Archaea (Persson et al., 1994), and because a moss, *Physcomitrella patens*, contains *tRNA-IPT* genes but not an *IPT* gene (Rensing et al., 2008; Yevdakova and von Schwartzenberg, 2007), IPT appears to be evolutionally diverged from tRNA-IPT. In future studies, it will be interesting to determine how IPT has evolved to use free adenine nucleotides as substrates for the direct synthesis of free CK-nucleotides.

6. Cytochrome P450 monooxygenases (CYP735As)

The iP-nucleotides produced by IPT in plants undergo hydroxylation at the prenyl side chain to synthesize tZ-nucleotides. In *Arabidopsis,* two cytochrome P450 monooxygenases, CYP735A1 and CYP735A2, catalyze the reaction (Takei et al., 2004b). The

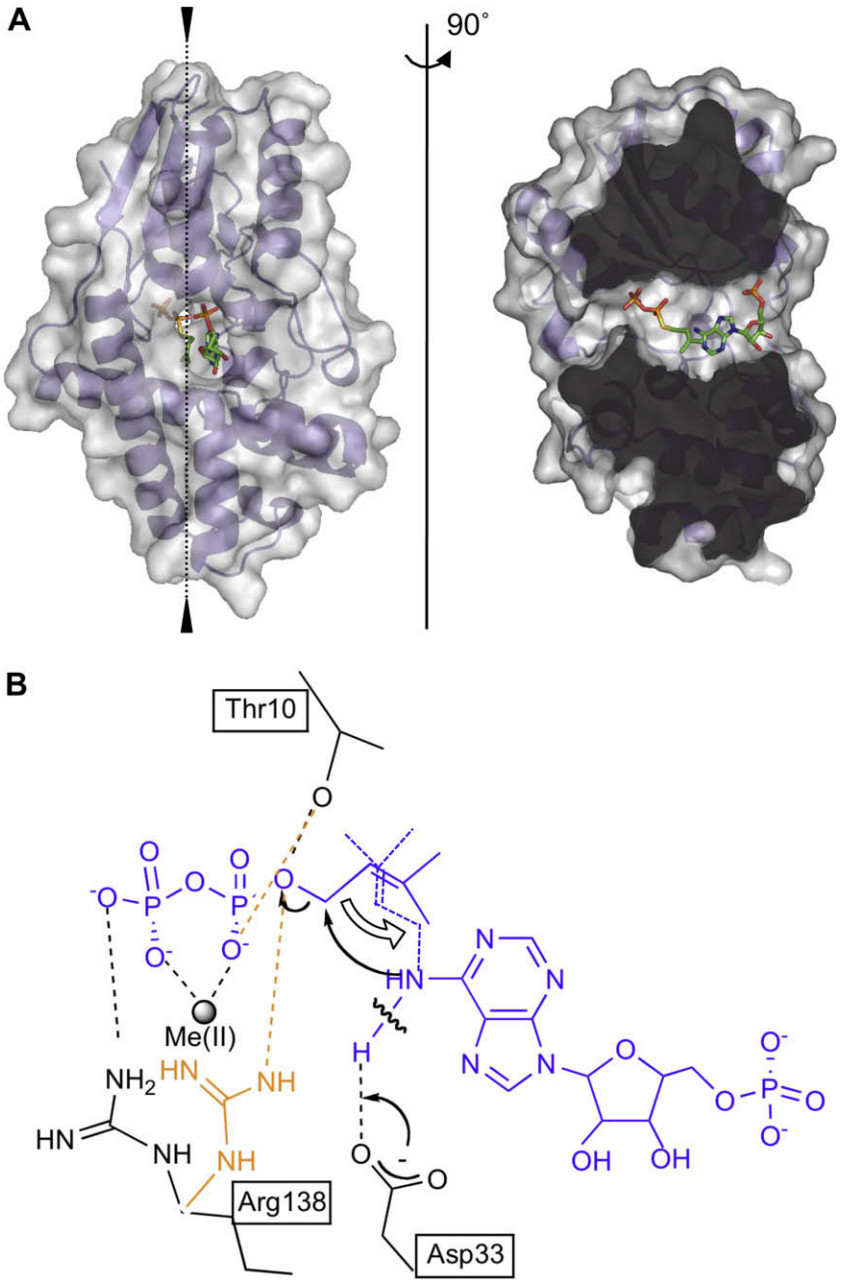


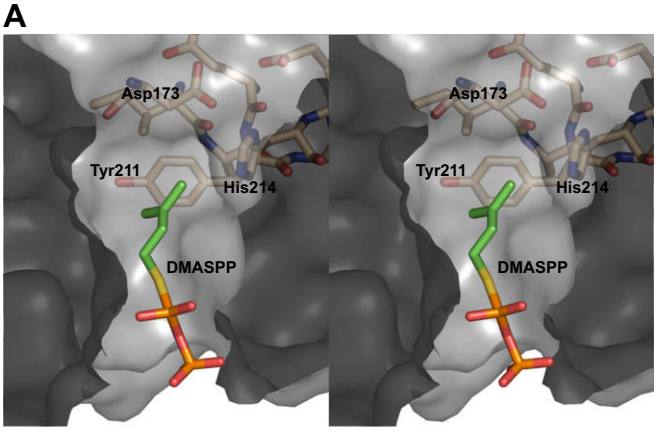
Fig. 2. (A) Surface model of Tzs complexed with AMP, DMASPP, and Zn^{2+}. This model is drawn with the atomic coordinates of Tzs-AMP/DAMSPP/Zn (PDB ID code, 2ZE7). DMASPP, dimethylallyl *S*-thiodiphosphate, is a substrate analog of DMAPP. Molecular surface of the AMP-binding side (left) and the 90°-rotated site (right) around the vertical axis (solid line). To see the reaction center channel, the protein molecule is cut at the broken line. Cross-sectional surfaces are dark grey. (B) A chemical reaction model of prenyl transfer from DMAPP to AMP. Substrates are shown in blue, and the amino acid residues of Tzs in black. Arg138 in the absence of divalent metal ion [Me(II)] is shown in orange. The hydrogen-bonding network among the substrates and Thr10, Asp33, Arg138, and Me(II) is indicated as a dashed line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CYP735As utilize iP-nucleotides, but not the iP-nucleoside or iP (Takei et al., 2004b) (Fig. 1). Since this reaction is stereo-specific, the CYP735As do not produce cZ-nucleotides (Takei et al., 2004b). Both CYP735A1 and CYP735A2 preferentially catalyze iPRMP and iPRDP rather than iPRTP (Takei et al., 2004b). The K_m values of CYP735A1 for iPRMP, iPRDP and iPRTP were 1.76, 2.28 and 8.32 μM, respectively, and those of CYP735A2 were 0.39, 0.76 and 4.9 μM, respectively.

Interestingly, *Physcomitrella* produces iP and cZ but less tZ, although the CK signaling pathway also function in this moss. Clear orthologs of *Arabidopsis* CYP735As are not found in *Physcomitrella in silico* analyses to date (Rensing et al., 2008). Further analyses are expected to reveal the details of CK biosynthesis in mosses that will provide additional insights into the evolution of the CK biosynthetic pathway and enzymes.

7. LOG

To become biologically active, CK-nucleotides produced by IPTs and CYP735As have to be converted to the free-base form. Recently, a CK-activating enzyme named LOG that directly converts CK-nucleotides to the active nucleobases was identified in rice (Kurakawa et al., 2007). LOG has phosphoribohydrolase activity and releases a ribose 5′-monophosphate moiety from CK-nucleo-



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   Tzs: (170)EDID(173) ---- (206)AIANEYLEHALSQ(218)
   Tmr: (169)KEID(172) ---- (205)GIAQEYFIHARQQ(217)
AtIPT1: (249)KAIG(252) ---- (286)DIKRNTWTLAKRQ(298)
AtIPT3: (209)KAIG(212) ---- (241)EIKRNTFELACRQ(253)
AtIPT4: (188)KAIG(191) ---- (225)AIKENTFQLTKDQ(237)
AtIPT5: (201)RAIG(204) ---- (234)KIKENTCLLACRQ(246)
AtIPT7: (202)RAIG(205) ---- (234)NIKKNTEILACRQ(246)
AtIPT8: (227)KTIG(230) ---- (266)EIKENTWRLAKKQ(278)
AtIPT2: (259)QSIG(262) ---- (318)RVKLNTRRLLRRQ(330)
AtIPT9: (321)RAIG(324) ---- (353)KFQTASRNFAKRQ(365)
  MiaA: (247)RCVG(250) ---- (270)RGVCATRQLAKRQ(282)
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Fig. 3. (A) Molecular surface of the reaction cavity surrounding the end of the dimethylallyl group. This model is drawn with the atomic coordinates of Tzs-AMP/DMASPP/Zn. Carbon, oxygen, phosphorous, and sulfur atoms of the DMASPP molecule are green, red, orange, and, yellow, respectively, whereas, carbon, oxygen, and nitrogen atoms of Tzs molecule are light-orange, red, and, blue, respectively. Sectional surfaces are dark grey. (B) Amino acid alignment of IPTs and tRNA-IPTs. Only the regions involved in forming the reaction cavity surrounding the end of the dimethylallyl group are shown. The amino acid residues affecting prenyl-donor substrate specificity in Tzs are red. Amino acid residue numbers are shown in the parentheses. MiaA, tRNA-IPT from *Escherichia coli* (accession no. BAE78172) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

side 5′-monophosphate (Kurakawa et al., 2007) (Fig. 1). All four CK-nucleoside 5′-monophosphates, iPRMP, tZRMP, DZRMP and cZRMP, are utilized by LOG, but the di- or triphosphate, AMP, or any CK-nucleosides are not suitable substrates. The K_m value of LOG for iPRMP and tZRMP are 11.7 and 22.0 μM, respectively (Kurakawa et al., 2007).

The name LOG comes from its mutant phenotype in which maintenance of the shoot meristem is defective and flowers often contain only one stamen but no pistil, thus *lonely guy* (Kurakawa et al., 2007). The phenotype clearly shows the importance of the LOG-dependent CK activation pathway in maintenance of shoot apical meristem in rice. When causal gene of *log* was initially cloned, its molecular function was unknown. The idea that LOG functions in CK metabolism resulted from finding homologous genes in soil phytopathogenic bacteria, *Agrobacterium rhizogenes* and *Rhodococcus fascians*. Both bacteria can produce CKs, and the LOG-homologous genes were closely located to IPT genes in each bacterium (Crespi et al., 1994; Moriguchi et al., 2001). Especially in *R. fascians*, genes of LOG homologue (*FAS6*) and IPT (*FAS4*) are co-transcribed as a single operon (Crespi et al., 1994). Genes with sequence similarity to LOG are also found in a number of other organisms; even in those that do not produce CKs, although their functions have not yet been investigated.

The notion that conversion from CK-nucleotides to the free-base forms requires a two-step enzymatic reaction catalyzed by nucleotidase and nucleosidase was advocated over 20 years ago (Chen and Kristopeit, 1981a,b). Enzymatic activities were identified in wheat germ, but the corresponding genes for nucleotidase and nucleosidase have not been identified to date. On the other hand, a significant amount of CK-nucleosides are actually detected in plants. An isotope labeling analysis showed that exogenous CK-nucleobases are converted to CK-nucleosides and CK-nucleotides (Letham, 1994). The report suggested that the CK-nucleosides might come from CK-nucleobases, although further exploration is required.

8. Concluding remarks

In this review, we summarized recent progress in the exploration of CK biosynthetic enzymes. According to the identification and characterization of enzymes functioning in CK biosynthesis, we have proposed an outline of the mechanism for CK biosynthesis, although some questions still remain. One significant matter is to reveal functional differences between each CK species, iP, tZ, cZ and DZ. CYP735As function in the hydroxylation of iP-nucleotides to tZ-nucleotides, thus CYP735As might play an important role in adjusting the levels of iP and tZ.

The structural analyses of *Agrobacterium* IPT revealed the reaction mechanism at the atomic level and elucidated the mechanism distinguishing the prenyl-donor substrates with or without a hydroxyl group. Further analyses using this technique on the other CK biosynthetic enzymes, such as CYP735As and LOG, will reveal each reaction mechanism in more detail. Moreover, structural characterization will provide significant clues for the design of chemical regulators that inhibit or modulate CK biosynthetic activity *in planta*. Those regulators of CK biosynthetic pathway will have advantages not only in biological studies but also in agricultural industry.

References

Akiyoshi, D.E., Klee, H., Amasino, R.M., Nester, E.W., Gordon, M.P., 1984. T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. Proc. Natl. Acad. Sci. USA 81, 5994–5998.

Barry, G.F., Rogers, S.G., Fraley, R.T., Brand, L., 1984. Identification of a cloned cytokinin biosynthetic gene. Proc. Natl. Acad. Sci. USA 81, 4776–4780.

Bilyeu, K.D., Cole, J.L., Laskey, J.G., Riekhof, W.R., Esparza, T.J., Kramer, M.D., Morris, R.O., 2001. Molecular and biochemical characterization of a cytokinin oxidase from maize. Plant Physiol. 125, 378–386.

Blackwell, J.R., Horgan, R., 1993. Cloned *Agrobacterium tumefaciens ipt1* gene product, DMAPP:AMP isopentenyl transferase. Phytochemistry 34, 1477–1481.

Chen, C.-M., Kristopeit, S.M., 1981a. Metabolism of cytokinin: dephosphorylation of cytokinin ribonucleotide by 5′-nucleotidases from wheat germ cytosol. Plant Physiol. 67, 494–498.

Chen, C.-M., Kristopeit, S.M., 1981b. Metabolism of cytokinin: deribosylation of cytokinin ribonucleoside by adenosine nucleosidase from wheat germ. Plant Physiol. 68, 1020–1023.

Crespi, M., Messens, E., Caplan, A.B., van Montagu, M., Desomer, J., 1992. Fasciation induction by the phytopathogen *Rhodococcus fascians* depends upon a linear plasmid encoding a cytokinin synthase gene. EMBO J. 11, 795–804.

Crespi, M., Vereecke, D., Temmerman, W., Van Montagu, M., Desomer, J., 1994. The *fas* operon of *Rhodococcus fascians* encodes new genes required for efficient fasciation of host plants. J. Bacteriol. 176, 2492–2501.

Faiss, M., Zalubilova, J., Strnad, M., Schmulling, T., 1997. Conditional transgenic expression of the *ipt* gene indicates a function for cytokinins in paracrine signaling in whole tobacco plants. Plant J. 12, 401–415.

Galuszka, P., Popelkova, H., Werner, T., Frebortova, J., Pospisilova, H., Mik, V., Kollmer, I., Schmulling, T., Frebort, I., 2007. Biochemical characterization of cytokinin oxidases/dehydrogenases from *Arabidopsis thaliana* expressed in *Nicotiana tabacum* L.. J. Plant Growth Regul. 26, 255–267.

Gan, S., Amasino, R.M., 1995. Inhibition of leaf senescence by autoregulated production of cytokinin. Science 270, 1986–1988.

Higuchi, M., Pischke, M.S., Mahonen, A.P., Miyawaki, K., Hashimoto, Y., Seki, M., Kobayashi, M., Shinozaki, K., Kato, T., Tabata, S., Helariutta, Y., Sussman, M.R., Kakimoto, T., 2004. *In planta* functions of the *Arabidopsis* cytokinin receptor family. Proc. Natl. Acad. Sci. USA 101, 8821–8826.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K., Kakimoto, T., 2001. Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. Nature 409, 1060–1063.

Kakimoto, T., 2001. Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate:ATP/ADP isopentenyltransferases. Plant Cell Physiol. 42, 677–685.

Kaminek, M., Paces, V., Corse, J., Challice, J.S., 1979. Effect of stereospecific hydroxylation of N6-(delta-2-isopentenyl)adenosine on cytokinin activity. Planta 145, 239–243.

Kasahara, H., Takei, K., Ueda, N., Hishiyama, S., Yamaya, T., Kamiya, Y., Yamaguchi, S., Sakakibara, H., 2004. Distinct isoprenoid origins of *cis*- and *trans*-zeatin biosyntheses in *Arabidopsis*. J. Biol. Chem. 279, 14049–14054.

Kim, H.J., Ryu, H., Hong, S.H., Woo, H.R., Lim, P.O., Lee, I.C., Sheen, J., Nam, H.G., Hwang, I., 2006. Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in *Arabidopsis*. Proc. Natl. Acad. Sci. USA 103, 814–819.

Krall, L., Raschke, M., Zenk, M.H., Baron, C., 2002. The Tzs protein from *Agrobacterium tumefaciens* C58 produces zeatin riboside 5′-phosphate from 4-hydroxy-3-methyl-2-(*E*)-butenyl diphosphate and AMP. FEBS Lett. 527, 315–318.

Kurakawa, T., Ueda, N., Maekawa, M., Kobayashi, K., Kojima, M., Nagato, Y., Sakakibara, H., Kyozuka, J., 2007. Direct control of shoot meristem activity by a cytokinin-activating enzyme. Nature 445, 652–655.

Laureys, F., Dewitte, W., Witters, E., van Montagu, M., Inze, D., van Onckelen, H., 1998. Zeatin is indispensable for the G2-M transition in tobacco BY-2 cells. FEBS Lett. 426, 29–32.

Laureys, F., Smets, R., Lenjou, M., van Bockstaele, D., Inze, D., van Onckelen, H., 1999. A low content in zeatin type cytokinins is not restrictive for the occurrence of G1/S transition in tobacco BY-2 cells. FEBS Lett. 460, 123–128.

Letham, D.S., 1994. Cytokinins as phytohormones-sites of biosynthesis, translocation, and function of translocated cytokinin. In: Mok, D.W.S., Mok, M.C. (Eds.), Cytokinins Chemistry, Activity, and Function. CRC press, Florida, pp. 57–80.

Malito, E., Coda, A., Bilyeu, K.D., Fraaije, M.W., Mattevi, A., 2004. Structures of Michaelis and product complexes of plant cytokinin dehydrogenase: implications for flavoenzyme catalysis. J. Mol. Biol. 341, 1237–1249.

Miyawaki, K., Matsumoto-Kitano, M., Kakimoto, T., 2004. Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: tissue specificity and regulation by auxin, cytokinin, and nitrate. Plant J. 37, 128–138.

Miyawaki, K., Tarkowski, P., Matsumoto-Kitano, M., Kato, T., Sato, S., Tarkowska, D., Tabata, S., Sandberg, G., Kakimoto, T., 2006. Roles of *Arabidopsis* ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. Proc. Natl. Acad. Sci. USA 103, 16598–16603.

Mok, D.W., Mok, M.C., 2001. Cytokinin metabolism and action. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 89–118.

Moriguchi, K., Maeda, Y., Satou, M., Hardayani, N.S., Kataoka, M., Tanaka, N., Yoshida, K., 2001. The complete nucleotide sequence of a plant root-inducing (Ri) plasmid indicates its chimeric structure and evolutionary relationship between tumor-inducing (Ti) and symbiotic (Sym) plasmids in *Rhizobiaceae*. J. Mol. Biol. 307, 771–784.

Morris, R.O., 1986. Genes specifying auxin and cytokinin biosynthesis in phytopathogens. Annu. Rev. Plant Physiol. Plant Mol. Biol. 37, 509–538.

Murai, N., 1994. Cytokinin biosynthesis in tRNA and cytokinin incorporation into plant RNA. In: Mok, D.W.S., Mok, M.C. (Eds.), Cytokinins: Chemistry, Activity, and Function. CRC Press, Boca Raton, Florida, pp. 87–99.

Nishimura, C., Ohashi, Y., Sato, S., Kato, T., Tabata, S., Ueguchi, C., 2004. Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in *Arabidopsis*. Plant Cell 16, 1365–1377.

Ordog, V., Stirk, W.A., van Staden, J., Novak, O., Strnad, M., 2004. Endogenous cytokinins in three genera of microalgae from the chlorophyta. J. Phycol. 40, 88–95.

Persson, B.C., Esberg, B., Olafsson, O., Bjork, G.R., 1994. Synthesis and function of isopentenyl adenosine derivatives in tRNA. Biochimie 76, 1152–1160.

Rensing, S.A., Lang, D., Zimmer, A.D., Terry, A., Salamov, A., Shapiro, H., Nishiyama, T., Perroud, P.F., Lindquist, E.A., Kamisugi, Y., Tanahashi, T., Sakakibara, K., Fujita, T., Oishi, K., Shin, I.T., Kuroki, Y., Toyoda, A., Suzuki, Y., Hashimoto, S., Yamaguchi, K., Sugano, S., Kohara, Y., Fujiyama, A., Anterola, A., Aoki, S., Ashton, N., Barbazuk, W.B., Barker, E., Bennetzen, J.L., Blankenship, R., Cho, S.H., Dutcher, S.K., Estelle, M., Fawcett, J.A., Gundlach, H., Hanada, K., Heyl, A., Hicks, K.A., Hughes, J., Lohr, M., Mayer, K., Melkozernov, A., Murata, T., Nelson, D.R., Pils, B., Prigge, M., Reiss, B., Renner, T., Rombauts, S., Rushton, P.J., Sanderfoot, A., Schween, G., Shiu, S.H., Stueber, K., Theodoulou, F.L., Tu, H., van de Peer, Y., Verrier, P.J., Waters, E., Wood, A., Yang, L., Cove, D., Cuming, A.C., Hasebe, M., Lucas, S., Mishler, B.D., Reski, R., Grigoriev, I.V., Quatrano, R.S., Boore, J.L., 2008. The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. Science 319, 64–69.

Romanov, G.A., Lomin, S.N., Schmulling, T., 2006. Biochemical characteristics and ligand-binding properties of *Arabidopsis* cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. J. Exp. Bot. 57, 4051–4058.

Sachs, T., Thimann, K.V., 1967. The role of auxin and cytokinins in the release of buds from dominance. Amer. J. Bot. 54, 134–144.

Sakakibara, H., 2004. Cytokinin biosynthesis and metabolism. In: Davies, P.J. (Ed.), Plant Hormones: Biosynthesis, Signal Transduction, Action! Springer, Dordrecht, pp. 95–114.

Sakakibara, H., 2006. Cytokinins: activity, biosynthesis, and translocation. Annu. Rev. Plant Biol. 57, 431–449.

Sakakibara, H., Kasahara, H., Ueda, N., Kojima, M., Takei, K., Hishiyama, S., Asami, T., Okada, K., Kamiya, Y., Yamaya, T., Yamaguchi, S., 2005. *Agrobacterium tumefaciens* increases cytokinin production in plastids by modifying the biosynthetic pathway in the host plant. Proc. Natl. Acad. Sci. USA 102, 9972–9977.

Sakamoto, T., Sakakibara, H., Kojima, M., Yamamoto, Y., Nagasaki, H., Inukai, Y., Sato, Y., Matsuoka, M., 2006. Ectopic expression of KNOTTED1-like homeobox protein induces expression of cytokinin biosynthesis genes in rice. Plant Physiol. 142, 54–62.

Sakano, Y., Okada, Y., Matsunaga, A., Suwama, T., Kaneko, T., Ito, K., Noguchi, H., Abe, I., 2004. Molecular cloning, expression, and characterization of adenylate isopentenyltransferase from hop (*Humulus lupulus* L). Phytochemistry 65, 2439–2446.

Samuelson, M.E., Larsson, C.-M., 1993. Nitrate regulation of zeatin riboside levels in barley roots: effects of inhibitors of N assimilation and comparison with ammonium. Plant Sci. 93, 77–84.

Shimizu-Sato, S., Tanaka, M., Mori, H., 2009. Auxin-cytokinin interactions in the control of shoot branching. Plant Mol. Biol. 69, 429–435.

Spichal, L., Rakova, N.Y., Riefler, M., Mizuno, T., Romanov, G.A., Strnad, M., Schmulling, T., 2004. Two cytokinin receptors of *Arabidopsis thaliana*, CRE1/AHK4 and AHK3, differ in their ligand specificity in a bacterial assay. Plant Cell Physiol., 1299–1305.

Strnad, M., 1997. The aromatic cytokinins. Physiol. Plant 101, 674–688.

Sugawara, H., Ueda, N., Kojima, M., Makita, N., Yamaya, T., Sakakibara, H., 2008. Structural insight into the reaction mechanism and evolution of cytokinin biosynthesis. Proc. Natl. Acad. Sci. USA 105, 2734–2739.

Suzuki, T., Miwa, K., Ishikawa, K., Yamada, H., Aiba, H., Mizuno, T., 2001. The *Arabidopsis* sensor His-kinase, AHK4, can respond to cytokinins. Plant Cell Physiol. 42, 107–113.

Takei, K., Sakakibara, H., Sugiyama, T., 2001a. Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in *Arabidopsis thaliana*. J. Biol. Chem. 276, 26405–26410.

Takei, K., Sakakibara, H., Taniguchi, M., Sugiyama, T., 2001b. Nitrogen-dependent accumulation of cytokinins in root and the translocation to leaf: implication of cytokinin species that induces gene expression of maize response regulator. Plant Cell Physiol. 42, 85–93.

Takei, K., Ueda, N., Aoki, K., Kuromori, T., Hirayama, T., Shinozaki, K., Yamaya, T., Sakakibara, H., 2004a. *AtIPT3* is a key determinant of nitrate-dependent cytokinin biosynthesis in *Arabidopsis*. Plant Cell Physiol. 45, 1053–1062.

Takei, K., Yamaya, T., Sakakibara, H., 2004b. *Arabidopsis CYP735A1* and *CYP735A2* encode cytokinin hydroxylases that catalyze the biosynthesis of *trans*-zeatin. J. Biol. Chem. 279, 41866–41872.

Tanaka, M., Takei, K., Kojima, M., Sakakibara, H., Mori, H., 2006. Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. Plant J. 45, 1028–1036.

von Schwartzenberg, K., Nunez, M.F., Blaschke, H., Dobrev, P.I., Novak, O., Motyka, V., Strnad, M., 2007. Cytokinins in the bryophyte *Physcomitrella patens*: analyses of activity, distribution, and cytokinin oxidase/dehydrogenase overexpression reveal the role of extracellular cytokinins. Plant Physiol. 145, 786–800.

Werner, T., Motyka, V., Strnad, M., Schmulling, T., 2001. Regulation of plant growth by cytokinin. Proc. Natl. Acad. Sci. USA 98, 10487–10492.

Werner, T., Motyka, V., Laucou, V., Smets, R., van Onckelen, H., Schmulling, T., 2003. Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15, 2532–2550.

Xie, W., Zhou, C., Huang, R.H., 2007. Structure of tRNA dimethylallyltransferase: RNA modification through a channel. J. Mol. Biol. 367, 872–881.

Yamada, H., Suzuki, T., Terada, K., Takei, K., Ishikawa, K., Miwa, K., Yamashino, T., Mizuno, T., 2001. The *Arabidopsis* AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. Plant Cell Physiol. 42, 1017–1023.

Yevdakova, N.A., von Schwartzenberg, K., 2007. Characterisation of a prokaryote-type tRNA-isopentenyltransferase gene from the moss *Physcomitrella patens*. Planta 226, 683–695.

Yonekura-Sakakibara, K., Kojima, M., Yamaya, T., Sakakibara, H., 2004. Molecular characterization of cytokinin-responsive histidine kinases in maize: differential ligand preferences and response to *cis*-zeatin. Plant Physiol. 134, 1654–1661.

Zhou, C., Huang, R.H., 2008. Crystallographic snapshots of eukaryotic dimethylallyltransferase acting on tRNA: insight into tRNA recognition and reaction mechanism. Proc. Natl. Acad. Sci. USA 105, 16142–16147.

Zubko, E., Adams, C.J., Machaekova, I., Malbeck, J., Scollan, C., Meyer, P., 2002. Activation tagging identifies a gene from *Petunia hybrida* responsible for the production of active cytokinins in plants. Plant J. 29, 797–808.